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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,388,441**, on June 10, 2002, by **WEI-PING MIN, THOMAS ICHIM AND JONATHAN HILL**, for "Immunomodulation Using RNA Interference".

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IMMUNOMODULATION USING RNA INTERFERENCE

ABSTRACT

Disclosed are novel methods of altering immune system function in a mammal by the administration of double stranded RNA with homology to genes of immunological importance. Said genes include cytokines, adhesion molecules and costimulatory molecules. More specifically, a method of altering immune system function through administration of modified genetically modified dendritic cells is disclosed.

DESCRIPTION

FIELD OF INVENTION

The invention teaches methods of altering the immune system in a mammal. Said alterations can be useful in the treatment of transplant rejection, autoimmune disease and cancer. Moreover, described is a method of producing a tolerogenic dendritic cell by modifying gene expression of dendritic cells in vitro or in vivo. Said tolerogenic dendritic cells can be used to inhibit autoimmunity and transplant rejection.

BACKGROUND OF INVENTION

Dendritic cells (DC) are the most potent antigen presenting cell (APC), having the ability to activate naïve and memory T cells about 100-fold higher than other APC such as B cells or macrophages (1, 2). A crucial role for DC in transplantation rejection is well established: graft DC directly stimulate recipient T cells to attack the transplanted organ, or in other situations, recipient DC endocytose donor antigen and activate recipient anti-graft responses (3). DC are also involved in the antigen-specific suppression of immune responses. This subset of DC, called tolerogenic DC (Tol-DC) have a distinct phenotype, suppress activation of conventional T cells, and activate T regulatory cells (Treg) in an antigen-specific manner (4-7). Tol-DC possess two distinguishing features: 1) Reduced expression of the co-stimulatory molecules CD40, CD80, and CD86 and 2) Reduced ability to secrete T cell activating cytokines such as interleukin-12. The relevance of the reduced costimulatory molecule expression is seen in studies were the DC-T cell interaction is blocked in order to prevent T cell activation. Blocking the CD40 ligand-CD40 interaction with antibodies leads to prevention of acute renal rejection in non-human primates (8). Blocking the interactions of CD80 and CD86 on DC with the T cell molecule, CD28 using CTLA4-Ig also results in the inhibition of graft rejection (9). From the perspective of cytokines, IL-12 is expressed in rejecting organs, whereas tolerance is associated with low expression of IL-12 on intra-organ DC (10).

Various agents have been described in the literature, which inhibit maturation of DC and promote tolerance. These agents induce the generation of DC that express lower levels of

costimulatory molecules. N-benzyloxycarbonyl-Ile-Glu(O-tert-butyl)-Ala-leucinal (PSI) is a proteosome inhibitor that blocks NF-KB activation and results in the in vitro production of tolerogenic DC (11). N-acetylcysteine is an antioxidant which similarly blocks NF-KB activation and generates immature, tolerogenic dendritic cells (12). Vitamin D3 also inhibits dendritic cell maturation and leads to production of tolerogenic dendritic cells (13). A common theme for tolerogenic DC is an immature state exemplified by suppressed expression of costimulatory molecules and IL-12.

Gene-specific targeting offers the ability to selectively suppress expression of individual genes without effecting whole cell function. At present, the only practical method of blocking gene expression involved treatment of cells with anti-sense oligonucleotides. This approach has been attempted in models of transplantation for blocking IL-2 production (14). Unfortunately, a relatively high concentration of antisense DNA must be transfected into the cell to obtain suppression, these concentrations are very difficult to achieve in vivo.

Blocking gene expression has also been described using ribozymes, which are catalytic RNAs (15). Targeting of androgen receptors using hammerhead ribozyme technology inhibits proliferation of both androgen-dependent and independent cancer cell lines (16). Unfortunately, delivery of ribozymes into the cells is difficult, and presently this is impossible in the clinical setting.

Specific silencing of genes using small interfering RNA (siRNA) is a newly developed method which is 1000 fold more potent than using antisense oligonucleotides. siRNA is a 21 base-duplex RNA that when added with a transfection agent to cell cultures, acts in concert with cellular components to silence the gene with sequence homology to one of the siRNA sequences (17). Originally described in plants, the biological basis for gene specific suppression of siRNA are due to a phenomenon called RNA interference. This interference effect is very potent and in some systems silencing occurs even after 6 cell divisions (18). In 2001, the first experiment assessing siRNA was performed in mammalian cells (19). Potent suppression of endogenous genes was mediated by

administration of picomolar quantities of double-stranded RNA. The effect seen in mammalian cells was potent (requiring only picomolar concentrations of siRNA), specific (only genes sharing sequence identity with the siRNA are affected) and long lasting (gene silencing can be passed on for multiple cell divisions). With exception to the results presented, siRNA mediated gene silencing has never before been attempted in immune cells.

Uniqueness of the disclosed invention resides in our discovery that cells of the immune system are susceptible to the gene-specific blocking activities of siRNA. Furthermore, the ability of a dendritic cell manipulated to stop expression of genes associated with rejection will is a specific embodiment of our discovery in that these altered dendritic cells can be used for blocking immune response of against transplanted organs or autoantigens.

SUMMARY OF THE INVENTION

Described are methods of altering immune response through administration into a purified cell population, or administration in vivo of an RNA duplex which possesses homology to one of the genes involved in immune response. By altering gene expression in immune cells, specific functions of the cells can be altered in order to prevent, ameliorate, or cure diseases associated with immune response.

An important embodiment of the disclosed invention involves generation of tolerogenic dendritic cells. Dendritic cells possess both immune stimulatory and immune suppressive molecules which are critical in determining the phenotype of the T cell after encounter with the dendritic cell. Blockade of stimulatory molecules on dendritic cells gives them the ability to induce Th2 and T suppressor cells, from naïve T cells. By using siRNA to block immune stimulatory molecules, the artificially generated tolerogenic dendritic cell can be used to block immune system activation in an antigen specific and antigen non-specific manner. Tolerogenic dendritic cells can be used therapeutically for diseases in which T cell activation is desired.

Another aspect of the invention involves blockade of inhibitory molecules in situations where immune enhancement is desired. Systemic and cell specific blockade of immune suppressive molecules by treatment with siRNA is within the scope of the disclosed invention.

DETAILED DESCRIPTION OF THE INVENTION

Methods of immune modulation are described by systemically blocking gene expression, by cell-specific inhibition of gene expression in vivo, and by blocking gene expression ex vivo in purified immune cells which can be re-introduced into the subject for immunological effects.

A method of practicing the disclosed invention involves blocking expression of the inflammatory cytokine TNF-a. Presently the only methods of suppressing production of this cytokine is through antibodies, and soluble receptors. Both of these methods are only partly effective and require continual administration.

siRNA to TNF-a can be produced using the method described by Tuschl T et al (18,20). According to this method, 21 nucleotide base-pair sequences are chemically synthesized using a new 5′-silyl protecting group in conjunction with a unique acid-labile 2′-orthoester protecting group, 2′-bis(acetoxyethoxy)-methyl ether (2′-ACE). The 2′-protecting groups are rapidly (<30 minutes) and completely removed under mild conditions in aqueous buffers. This method, called "2′-ACE™ technology" was developed by Dharmacon Inc (Lafayette, CO, USA) and enables the routine synthesis of RNA oligonucleotides in high yield.

Admixing various concentrations of siRNA specific to TNF-a with an agent that crosses the cell membrane and enters the nucleus may be necessary to achieve maximal inhibition of TNF-a, such agents include cationic and anionic liposomes, as well as compositions of chemicals which permit transmembrane entrance of the siRNA without affecting the function of the nucleotides. In addition to compounds which allow entry of siRNA into the cell, the siRNA may be mixed with pharmaceutically acceptable carrier molecules which allow for the stability of siRNA and the transmembrane crossing agent when they are administered into systemic circulation. Pharmaceutically acceptable carriers may include, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

The therapeutic mixture, containing siRNA, and/or an agent that allows for transmembrane crossing of the siRNA, and/or a pharmaceutically acceptable carrier can be administered to a mammal in need by the intravenous route, subcutaneous route, oral route, intrarectal route, or transdermally. The optimal method of administration is dependent upon the area of the body where suppression of TNF-a is most desired, as well

as the health and disease-state of the patient. For diseases associated with systemic rises in TNF-a, the dosage of siRNA administered can be guided by serum ELISA measurements for levels of this cytokine. In patients where systemic intravenous administration is required, siRNA can be infused via a portable volumetric infusion pump (Block Medical, Carlsbad, CA) at a rate between 1 and 6 mL/hour depending on the volume to be infused. Final doses of 0.1 mg/kg/day-10 mg/kg/day may be used for the necessary time period until suppression of TNF-a is achieved.

Suppression of TNF-a is desirable in many disease states, these include sceptic shock, rheumatoid arthritis, transplant rejection, scleroderma, immune mediated diabetes, chronic inflammatory bowel syndrome, HIV, cancer, cholitis, crohn's dosease and inflammation of chronic illness. However, the described invention encompasses treating other diseases mediated by other cytokines. For example, in an immune mediated inflammatory disease other cytokines may contribute to pathology if TNF-a is suppressed. These cytokines could be IL-1, IL-6, IFN-gamma, or lymphotoxin. The disclosed invention can be applied to treating diseases associated with these cytokines by targeting expression through administration of effective siRNA compositions.

Another embodiment of the invention is a method of generating tolerogenic dendritic cells (DC). As discussed in the background, DC possess activatory and inhibitory signals. By blocking expression of activitory signals, the inhibitory signals will block the activation of T cells, and instead lead to generation of regulatory T cells. These regulatory T cells are antigen-specific and block ability of T cells specific for the said antigen to be activated.

One method of generating tolerogenic DC is by blocking expression of IL-12. Using siRNA sequences that suppress expression of this gene, the DC loses ability to activate T cells, and instead promotes activation of Th2 and/or regulatory cells. For treatment of autoimmune diseases where the antigen is known, patient DC generated from bone marrow, or peripheral blood can be loaded with the autoantigen. These DC are then administered siRNA for IL-12 and subsequently re-infused into the patient. These DC will only generate T regulatory cells and/or Th2 cells specific for the autoantigen. Similar methods modifying DC to induce

tolerance to an autoantigen were described by Morita et al who transfected DC with the inhibitory cytokine IL-4 and showed that their administration blocked progression of collagen-induced arthritis (21). In contrast to Morita et al, our tolerogenic DC can be generated in vivo. By using an immunoliposome specific to DC, targeting a DC-specific surface molecule such as DEC-205, CD11c, or CD83, the siRNA can be administered in systemically in vivo, in such a manner to target DC in homeostatic conditions.

EXAMPLES

Example I Suppression of IL-12 Production From Bone Marrow Derived DC

The procedure used in our laboratory to generate DC from bone marrow-derived precursor cells is similar to that first described by Inaba *et al* (22). Bone marrow cells were flushed from the femur and tibia of C57BL/6 mice, washed and cultured in 24-well plates (2 x 10^6 cells per well) in 2 ml RPMI 1640 supplemented with 10% fetal-calf serum (FCS, Gibco RBL), 100 U/ml of penicillin, 100 µg of streptomycin, 2-mercaptoethanol (50 µM, Gibco RBL), recombinant GM-CSF (10 ng/ml; Peprotech, Rocky Hill, NJ) and recombinant mouse IL-4 (10 ng/ml; Peprotech). All media and additives were documented to be free of LPS contamination (23). Nonadherant granulocytes were removed after 48 hrs of culture and fresh media added every 48 hrs. By day 4-6 of culture, proliferating clusters of cells with typical dendritic morphology were seen. By day 7 of culture >90% of the cells expressed the DC specific marker DEC-205. The proportion of cells staining for T (CD3) and B (B220) lymphocytes was consistently <3%. DC were used for gene transfection on day 7 of culture.

6 picograms of siRNA specific for IL-12 with the sequence:

C.C.U . G.C.U . G.A.A. . G.A.C. . C.A.C. . A.G.A . U.dT.dT
dT.dT . G.G.A . C.G.A . C.U.U . C.U.G. . G.U.G. . U.C.U . A

were incubated with 8 µl of Lipofectin (Gibco RBL) or 4 µl of Lipofectamine (Gibco RBL) in a volume of 100 µl of PBS at room temperature for 45 min before addition to 7-day cultured DC in a 24-well plate, in a final volume of 1 ml of serum-free medium. After 4-hour of incubation at 37 °C with 5% CO_2, the cells were washed and cultured in RPMI 1640 with 10% FCS for 48 h. After the incubation period (DC day 9), LPS (100ng/ml), LPS + IFN-g, and 10^6 allogeneic (BALB/c origin) T cells were added to the DC. 48 hours later (DC day 11) supernatants were collected from the cultures and assessed for production of IL-12 using commercially available ELISA kits (R&D

Systems, Minnesota, USA). As seen in Figure 1, IL-12 production was completely inhibited in the cultures treated with the dsRNA specific for IL-12. No inhibition was seen in the cultures treated with the random dsRNA sequence.

Example 2. Blocking IL-12 Production With siRNA Induces DC2 and Tolerogenic Phenotype

Bone marrow derived DC were generated, transfected with siRNA specific for IL-12, and activated as described in Example I. Analysis of DC2 phenotype was performed by assessing the ability of DC to suppress Th1 cytokine production (IFN-g) and stimulate Th2 cytokine (IL-4) from T cells in mixed lymphocyte reaction. Additionally, another characteristic of DC2 and tolerogenic DC is ability to secrete IL-10. This was also tested.

DC activated for 12 hours with LPS (10ng/ml), DC were harvested and cytokine gene expression was detected by RT-PCR. RNA was isolated using the TRIzol reagent (Gibco RBL) as per the manufacturer's instructions. First strand cDNA was synthesized using an RNA PCR kit (Gibco RBL) with the supplied oligo d(T)16 primer. Reverse transcription was performed using a thermal program of RT 10 min; 42 °C 30 min; and 90 °C 10 min.

One microliter reverse transcription reaction product was used for the subsequent PCR reaction. Primers for murine IL-4, IL-10 and IFN-g were used. The PCR conditions used were 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, for 35 cycles. The PCT products of hFasL (293 bp) and β-actin (389 bp) were visualized with ethidium bromide on 1.5% agarose gel. As seen in Figure 2, DC treated with IL-12 siRNA possessed increased transcripts for IL-10 whereas IL-12 transcripts were absent. Addition of BALB/c T cells to C57BL/6 DC results in a mixed lymphocyte reaction with production of IFN-g and IL-4. Figure 3 demonstrates that DC treated with siRNA for IL-12 induced a potent stimulation of IL-4 secretion in mixed lymphocyte reaction. IL-4 was assessed by ELISA. In order to verify ability of the IL-12 siRNA treated DC to stimulate Th2 cytokine profile, RT-PCR was performed in T cells after MLR. Responding BALB/c T cells possessed increased IL-4 and decreased IFN-g transcripts

when IL-12 was knocked-down with siRNA. In contrast, control DC stimulated T cells to produce more IFN-g and less IL-4 (Figure 4).

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of altering immune response in a mammal through administration of double stranded RNA in which;
   a) One of the strands is identical to part or all of a gene involved in immune response whose suppression is desired,
   b) The sequence of double stranded RNA can but is not restricted to 21-36 nucleotides long,
   c) One of the strands in the double stranded RNA sequence may be identical to the sequence that begins 75 nucleotides downstream of the start codon for the gene whose suppression is targeted,
   d) The sequence for one of the strands in the double stranded RNA begins after the first adenine-adenine repeat of the exon for the gene whose suppression is desired.

2. The method of claim 1 where the double stranded RNA is administered through a lipid-based vector such as lipofectamine, lipofectin, oligofectamine, or GenePorter.

3. The method of claim 1 where the double stranded RNA is administered as part of a pharmaceutical preparation through the oral, intravenous, subcutaneous, intranasal, intraperitoneal, intramuscular, subrectal, vaginal, or intraocular route.

4. The method of claim 1 where the double stranded RNA is administered as a topical cream in a preparation that is readily absorbed through the dermis.

5. The method of claim 1 where one strand of the double stranded RNA possesses specific homology to part or to the whole exon region of a cytokine, costimulatory, adhesion molecule, or growth factor gene.

6. The method of claim 1 where one strand of the double stranded RNA is specific to part or to the whole exon region of a gene responsible for production of transcription factors.

7. A method of inhibiting the T cell activating ability of an antigen presenting cell by administrating to the said cell a quantity of double stranded RNA sufficient to inhibit production of the costimulatory molecules, cytokines, adhesion molecules and transcription factors.

8. The method of claim 7 where the antigen presenting cell is a macrophage, or a myeloid, lymphoid, or circulating blood dendritic cell.

9. The method of claim 7 where one strand of the double stranded RNA contains a sequence specific to the gene encoding for NF-κB, interleukin-12, CD40, CD80, CD86, MHC I, MHC II, ICAM-1, TRANCE, CD200 and CD200 receptor.

10. The method of claim 7 where the double stranded RNA is targeted to antigen presenting cells in vivo by administration of a pharmaceutical preparation that possesses preferential affinity for antigen presenting cells.

11. The method of claim 10 where the pharmaceutical preparation comprises of double stranded RNA admixed with a chemical agent that preferentially interacts with antigen presenting cells.

12. The method of claim 10 where the pharmaceutical preparation comprises an antibody-based targeting strategy in which the double stranded RNA is targeted to the antigen presenting cell via immunoliposomes, or antibody-double stranded RNA complexes.

13. A method of treating Th1 mediated autoimmune disease through administration of double stranded RNA specific for Th1 cytokines, in quantities necessary to inhibit production of these cytokines.

14. A method for blocking rejection of a transplanted allograft or xenograft by administration to the graft recipient sufficient quantities of double stranded RNA specific for genes involved in rejection of the graft.

15. The method of claim 14 whereas the genes associated with rejection of the graft are IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, IL-18, MIP-1α, MIP-1b, IFN-g, TNF-a, TRAIL, CD40, CD80, CD86, CD200, CD200 receptor, MHC I, MHC II, NF-kB, iNOS, arginase, FcgammaR I and III, and thrombin.

16. A method of decreasing immunogenicity, and rejection potential of an organ for transplantation by perfusing the organ with sufficient quantities of double stranded RNA such that inhibition of rejection associated genes occurs, said genes being IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, IL-18, MIP-1α, MIP-1b, IFN-g, TNF-a, TRAIL, CD40, CD80, CD86, CD200, CD200 receptor, MHC I, MHC II, NF-kB, iNOS, arginase, FcgammaR I and III, and thrombin.

17. A method for the generation of tolerogenic dendritic cells through administration of double stranded RNA specific for genes associated with immune activation.

18. The method of claim 17 where the tolerogenic dendritic cell can be used for treatment of autoimmune diseases and transplantation rejection.

19. The method of claim 17 where the genes associated with immune activation comprise of the genes encoding IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, IL-18, MIP-1α, MIP-1b, IFN-g, TNF-a, TRAIL, CD40, CD80, CD86, CD200, CD200 receptor, MHC I, MHC II, NF-kB, iNOS, arginase, FcgammaR I and III, and thrombin.

FIGURE I

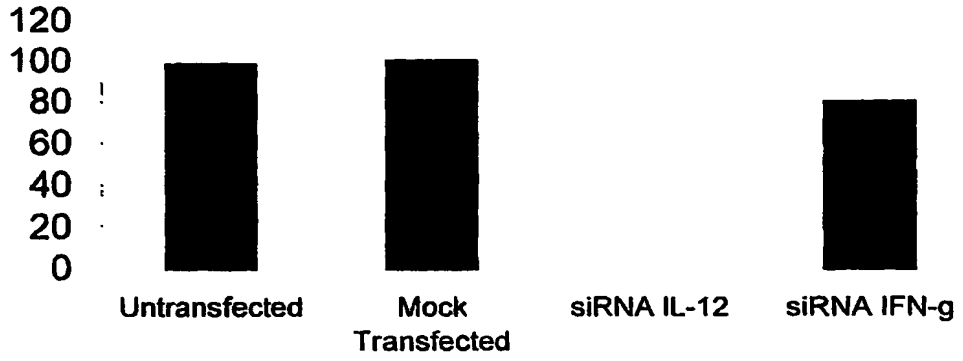
120
100
80
60
40
20
0
Untransfected
Mock Transfected
siRNA IL-12
siRNA IFN-g

FIGURE II

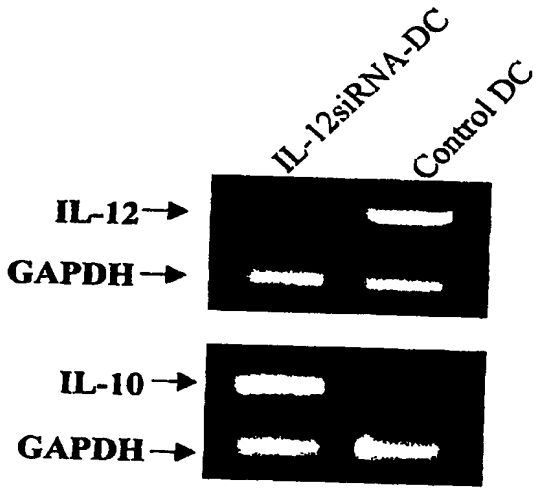

FIGURE III

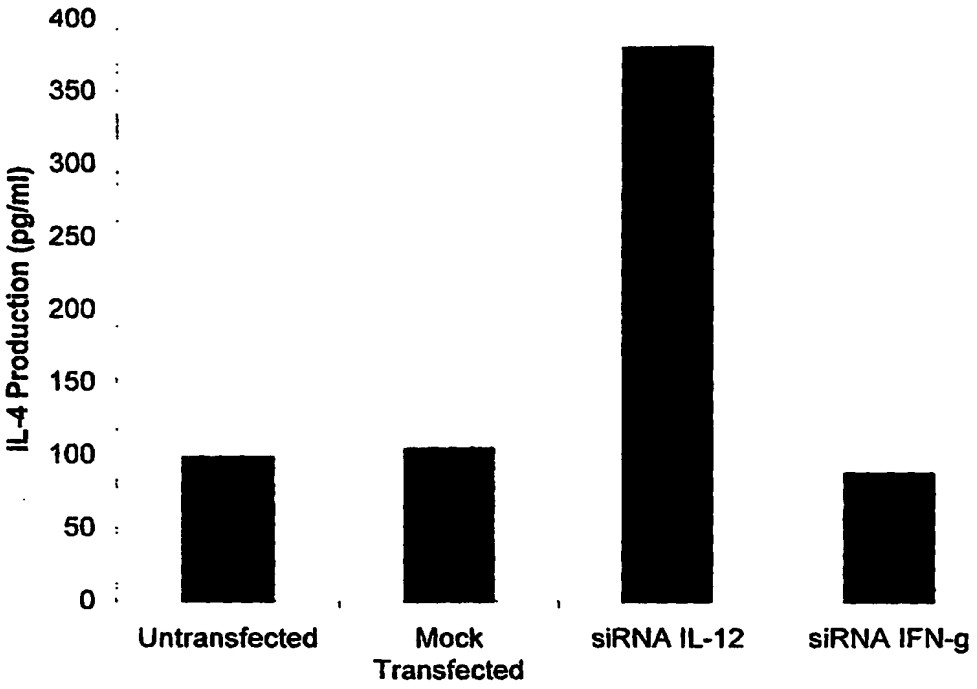
400
350
300
250
200
150
100
50
0
IL-4 Production (pg/ml)
Untransfected
Mock Transfected
siRNA IL-12
siRNA IFN-g

FIGURE IV